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Award Number: DAMD17-96-1-6134

TITLE: Role of Bone Sialoproteins in Osseous Metastasis of Breast Cancer

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Washington DC 20057

REPORT DATE: October 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

-REPORT DOCUMENTATION PAGE-

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Final (1 Jul 96 - 30 Sep 99)

4. TITLE AND SUBTITLE
Role of Bone Sialoproteins in Osseous Metastasis of Breast Cancer

5. FUNDING NUMBERS
DAMD17- 96-1-6134

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distributed unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Recently, antibodies to the growth factor receptor ErbB2 have been established as a valid therapeutic approach in the treatment of breast cancer. Because breast cancers can express a number of growth factor receptors the evaluation of antibodies targeting other growth factor receptors implicated in the progression of breast cancer is extremely important. We have explored the potential of blocking antibodies targeting the insulin-like growth factor 1 receptor (IGF1R) to inhibit the growth of breast cancer cells using an *in vitro* model. Limited dilution cloning of T47D:A18 cells in estrogen free media generated the panel of T47D:C4:*n*W clones. These cell lines were originally estrogen receptor negative. We assessed the status of ER and IGF1R in these cells lines and found them to be a useful model to determine the effects of IGF1R blocking antibodies.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Michele K Dougherty 10/29/99

PI - Signature Date

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The work proposed for the funding year involved determining the effects of insulin like growth factor 1 receptor (IGF1R) inhibitory antibodies 24-57 and 24-60 (1), in estrogen receptor (ER) positive, IGF1R positive MCF-7 cells. Shortly after beginning work on this project, our lab obtained a set of ER negative T47D clonal cell lines, designated T47D:C4:*n*W, and the parental ER positive T47D:A18 cell line (2). Since IGF1R expression can be regulated by estrogen receptor, we anticipated that T47D:C4:*n*W cells would no longer express IGF1R. This type of *in vitro* model would provide us with a valuable negative control for determining the effects of these antibodies on IGF1 mediated signaling. The goals for this project subsequently included determining 1) the status of the insulin like growth factor system in T47D:C4:*n*W cells, 2) the effects of IGF1 treatment in these cells, 3) the effect of antibody treatment on growth and survival of these cell lines in the presence of IGF1 and other growth factors.

Background

Insulin like growth factor 1 receptor (IGF1R) is a tetrameric transmembrane tyrosine kinase receptor. Ligands for IGF1R include IGF1, IGF2 and insulin (3). Ligand stimulation of the receptor facilitates interaction with a number of downstream effector molecules including Shc, Crk and the family of insulin receptor substrate (IRS) proteins (4, 5, 6, 7). Interaction of activated IGF1R with multiple downstream effectors suggests several cellular effects. Consistent with these observations, IGF1R has been shown to influence mitogenesis, apoptosis and cell motility (8, 9, 10, 11). The phenotype of IGF1R knockout mice suggests that the receptor plays a role in normal growth and development (12). Additionally, IGFs synergize with estrogen to facilitate normal mammary gland formation in a mouse model (13).

IGF1R activity is required *in vitro* for growth and maintaining the transformed phentoype. IGF1R-deficient fibroblasts exhibit a reduced growth rate and are resistant to transformation (14). IGF1 can positively regulate growth of MCF-7 breast cancer cells, and synergize with estrogen to generate a more potent mitogenic signal in these cells (15). Anti-apoptotic effects of IGF1 treatment have been observed in breast cancer cells treated with chemotherapeutic agents (16, 17). Numerous animal models have demonstrated the necessity of IGF1R activity for tumorigenesis (18, 19, 20, 21, 22). Therapeutic strategies targeting IGF1R, including antisense and antibody therapies (18, 19, 22), have shown efficacy in some of these models. These observations support the conclusion that IGF1R contributes to the growth and survival of cancer cells *in vitro* and *in vivo*.

IGF1R is expressed in numerous human cancers including breast, liver, skin and kidney. In breast cancer, IGF1R overexpression has been observed in 80% of cases (13, 23). IGF1R expression often associates with ER and progesterone receptor status and is therefore considered a favorable prognostic marker (23, 24, 25). However, IGF1R expression has also been observed in ER negative breast cancer and is associated with a decrease in overall survival (26, 27)]. The ability of IGF1R to mediate cell survival (anti-apoptotic) signals has generated the hypothesis that IGF1R may render ER negative IGF1R positive breast cancer cells less sensitive to the effects of cytotoxic chemotherapy (27, 28)]. These observations indicate IGF1R may contribute to growth and survival of both ER positive and ER negative breast cancer. Therapeutic intervention targeting IGF1R may prove beneficial for slowing tumor growth and increasing the efficacy of adjuvant chemotherapy.

Current Progress

T47D:C4:*n*W cells were generated in the laboratory of Dr. VC Jordan by limited dilution cloning of ER positive T47D cells in estrogen free conditions. The panel of clones was initially negative for expression of the estrogen receptor. During culture in media supplemented with 10% fetal bovine serum, some of the cell lines have re-expressed ER (Fig 1). Using RNase protection assays we have observed T47D:C4:9W and T47D:C4:2W (lane 5&6) cells maintain an ER negative phenotype during culture in 10% FBS. T47D:C4:12W and T47D:C4:8W (lane 3&4) cells exhibit intermediate levels of ER while T47D:C4:10W (lane 2) cells express ER at levels comparable to the parental T47D:A18 cells (lane 1). Consistent with these observations, all of the C4:*n*W clones have downregulated expression of IGF1R to levels undetectable by western blot, with the

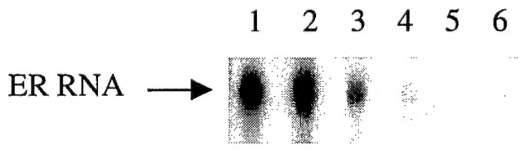

Figure 1. Estrogen receptor expression in T47D:C4:*n*W clones. RNase protection assay was used to determine levels of ER message in the panel of clones. Some of the cell lines have re-expressed ER during culture in the presence of estrogen (10% FBS) (lanes 2-4), while other cell lines remain negative for ER expression (lanes 5&6). Lane 1: T47D:A18, lane 2: T47D:C4:10W, lane 3: T47D:C4:12W, lane 4: T47D:C4:8W, lane 5: T47D:C4:9W, lane 6: T47D:C4:2W.

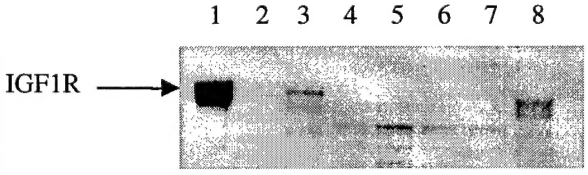

Figure 2. IGF1R expression in T47D:C4:*n*W breast cancer cells. IGF1R expression was detected with the 24-60 antibody on a western blot. IGF1R is absent in cells with low or no level of ER expression (lanes 4-7) but is evident in cells (lane 8) that have ER levels similar to parental cells (lane 3). Lane 1: NWT21 (NIH 3T3 cells engineered to overexpress IGF1R), lane 2: NIH3T3, lane 3: T47D:A18, lane 4: T47D:C4:2W, lane 5: T47D:C4:8W, lane 6:T47D:C4:9W, lane 7: T47D:C4:10W, lane 8: T47D:C4:12W.

exception of T47D:C4:12W cells (Fig 2, lane 8). Insulin receptor (IR) and insulin like growth factor 2 receptor (IGF2R) expression are similar among the parental cell line and C4:*n*W clones(data not shown). IRS-1 expression is downregulated in T47D:C4:*n*W clones (Fig 3) IRS-1 is a target for activated IGF1R, we have analyzed the activity of IGF1R following stimulation with IGF1 by monitoring tyrosine phosphorylation of IRS-1. Not surprisingly, IRS-1 phosphorylation is substantially decreased in ER negative T47D:C4:*n*W clones as compared to the parental T47D:A18 cells (Fig 3 compare lanes 3-7 with lane 2). These results suggest that IGF1R participates in growth regulation of

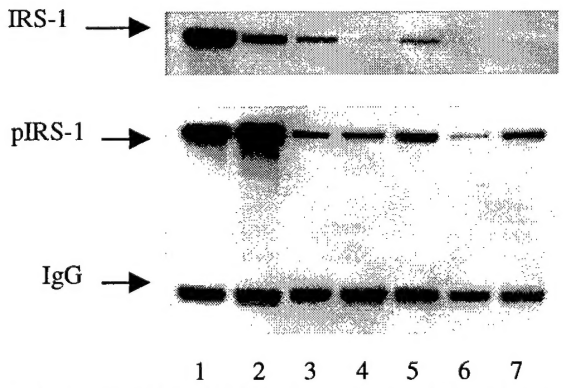

Figure 3. T47D:C4:*n*W clones express lower levels of IRS-1 and IRS-1 tyrosine phosphorylation is decreased in C4:*n*W clones following IGF1 stimulation. IRS-1 expression was detected by western blottin.g (upper panel). To detect IRS-1 tyrosine phosphorylation, cultures were serum starved 24 hours and stimulated with 10 nM IGF-1 for 5 minutes. IRS-1 protein was immunoprecipitated and phosphorylated protein was detected on a western blot with a monoclonal αphosphotyrosine antibody (lower panel). Consistent with the decrease in IGF1R expression, C4:*n*W clones (lanes 3-7) no longer respond to IGF1 stimulation as demonstrated by the decrease in IRS-1 phosphorylation following IGF1 stimulation.. Lane 1: NWT21, lane 2: T47D:A18, lane 3: T47D:C4:2W, lane 4: T47D:C4:8W, lane 5: T47D:C4:9W, lane 6: T47D:C4:10W, lane 7: T47D:C4:12W

T47D:A18 cells, while C4:*n*W clones are no longer dependent on IGF1R for growth or survival.

We have attempted to confirm that C4:*n*W cells no longer respond to the mitogenic effects of IGF1 or IGF2 using 96-well plate growth assays. These assays have been conducted in both serum free and low serum (0.1% FBS) conditions. However, we have not seen any differences in proliferation of T47D:A18 cells in the absence or presence of IGF in these conditions. Consequently, we are utilizing alternate methods to

confirm or refute these results. Currently, growth assays are being conducted in 24 well plates. Proliferation is assessed by counting cells in the presence of trypan blue to distinguish live cells and expressed as an increase in absolute cell number over time.

The 24-60 IGF1R inhibitory antibody was initially available as a crude ascites fluid. We utilized this preparation in experiments to evaluate the effect 24-60 on IRS-1 phosphorylation following IGF treatment (Fig. 4). These experiments were conducted in NWT21 cells, NIH 3T3 cells engineered to overexpress IGF1R. The results indicate

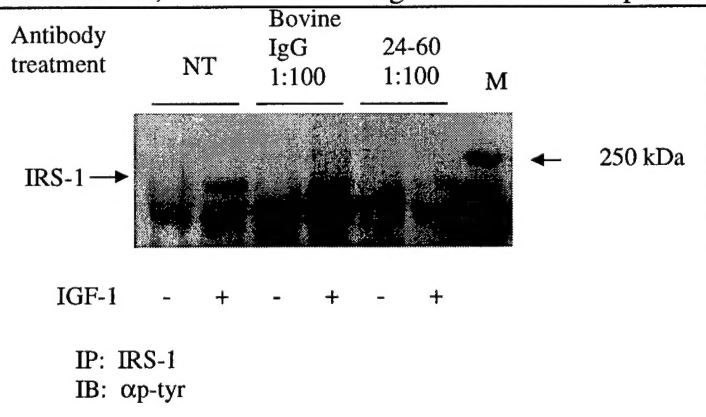

Figure 4. 24-60 antibody inhibits IGF1R activation. NWT21 cells were serum starved for 24 hours in the presence of 24-60 antibody, bovine IgG or no antibody treatment. Following stimulation with IGF1, IRS-1 was immunoprecipitated and analyzed for phosphotyrosine content. No IRS-1 phosphorylation was observed in cells treated with 24-60 antibody. 24-60 treatment in the absence of IGF also did not cause activation of IGF1R.

24-60 antibody can inhibit activation of the receptor in the presence of IGF1, suggesting this antibody can be an effective inhibitor of signaling through IGF1R. Significantly, no IRS-1 phosphorylation was observed in the absence of IGF1 stimulation suggesting that the antibody cannot activate the receptor. A collaboration with Novartis Pharmaceuticals will provide purified 24-60 antibody in milligram quantities allowing us to confirm and extend these results.

Conclusions and Future Directions

The data presented here describe an *in vitro* model that will be utilized to determine the effects of purified IGF1R inhibitory antibodies on growth and survival of

breast cancer cells. This model will allow us to determine the specificity of the antibodies for inhibiting IGF1R signaling pathways since it includes IGF1R positive and IGF1R negative cell lines from a similar genetic background. Preliminary results indicate the 24-60 antibody can efficiently block activation of IGF1R and should be further studied for its potential clinical application.

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Appendix I

Research Accomplishments

1) Demonstrated status of insulin like growth factor 1 receptor (IGF1R) expression and activation in parental T47D:A18 breast cancer cells and C4:*n*W ER negative, IGF1R negative clones.

2) Demonstrated IGF1R inhibitory potential of the 24-60 antibody in IGF1R over-expressing NWT21 cells.

Appendix II

Reportable Outcomes

1) Abstract and Poster presentation at 1999 AACR Meeting, Philadelphia, PA

Dougherty, M.K., Schumaker, L.M., Jordan, V.C., Ellis, M.J. Status of the insulin-like growth factor network in an estrogen receptor negative T47D breast cancer cell clone. Proceedings AACR 40, Abstract #4042, 1999.